a C^∞-diff. T of E onto E. The mapping T is characterized as follows. Let Int Z denote the smallest product of n subintervals of the respective coordinate axes of E which contains a bounded subset Z of E. Let $\boldsymbol{u}$ be the identity map of E. We define T so that

$$T|H'' = \boldsymbol{u}|H'', \qquad T|H' = R|H',$$
$$RT(K) \cap T(K) = 0, \qquad T(\mathring{K}) \subset \text{Int } [\mathring{K} \cup R\mathring{K}],$$

and so that under T the sign of the coordinate x_n remains invariant.

LEMMA 5.1. *The mapping* $\boldsymbol{k} = \boldsymbol{s}\,\boldsymbol{t}^{-1}\,\mathrm{I}$ *is a* C^∞*-diff. of* $E - P$ *onto* $\boldsymbol{X}^*$.

6. *Solution of a Problem of Type K.*—Let D be an n-interval in E, geometrically similar to H', containing P, and with the vertex (a) in common with H' $(a_i = -1;\ i = 1, \ldots, n)$. Let $D_1 \supset G'$ be a second n-interval, geometrically similar to H' and D, with vertex (a), and with $H' \supset \bar{D}_1$. Note that $D \supset D_1$. We define a C^∞-diff. $\boldsymbol{a}$ of D onto H', which leaves D_1 pointwise invariant. The open subsets

$$X = [M^* \cap D, \mathfrak{M}^*, \boldsymbol{X}^*], \qquad Y = [H' - G', \mathfrak{H}', \boldsymbol{X}^*],$$

of $\boldsymbol{X}^*$ are introduced, and proved to be μ^*-represented. The point $\boldsymbol{a}(P)$ is in $H' - G'$ and the point $Q = \pi_1^*[\boldsymbol{a}(P)]$ is in Y. With the aid of $\boldsymbol{a}$, a C^∞-diff. $\boldsymbol{D}$ of X onto $Y - Q$ is defined. The map

$$p \to (\pi_2^*)^{-1}[\boldsymbol{D}(\boldsymbol{k}[\boldsymbol{a}^{-1}(p)])] \qquad [p \in H' - \boldsymbol{a}(P)]$$

defines a C^∞-diff. of $H' - \boldsymbol{a}(P)$ into $\mathfrak{H}'$ and, if completed by mapping $\boldsymbol{a}(P) \to \omega[\boldsymbol{a}(P)]$ is a C_0^∞-diff. λ_ω of H' onto $\mathfrak{H}'$.

THEOREM 6.1. *Each problem of type K has a solution, thereby implying a solution of an arbitrary Schoenflies problem in Theorem* 1.1.

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TRANSFORMATION OF BIOCHEMICALLY DEFICIENT STRAINS OF BACILLUS SUBTILIS BY DEOXYRIBONUCLEATE

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INTRODUCTION

The experimental transfer of genetic characters by deoxyribonucleate (DNA) represents a powerful technique for the investigation of nucleic acid function at the molecular level.[1] The present study was initiated to obtain new microbial systems in which transformation of specific biochemical factors could be readily studied. By employing germinating spores of nutritionally deficient strains

of *Bacillus subtilis*, it was found that DNA isolated from a wild-type strain can transform these bacteria to nutritional independence. These new systems provide simple models for the study of the mechanism of genetic determinations and the biochemical control of enzyme synthesis.

METHODS

Strains of B. subtilis.—The strains of *B. subtilis* were obtained from Dr. Charles Yanofsky and originated from a collection of Burkholder and Giles[2] at Yale University.

Wild-type *B. subtilis* 23[3] was employed for the isolation of transforming DNA. The following strains of nutritionally deficient *B. subtilis* were used as recipients for the transformation studies:

Strain No.	Growth Requirement
168	L-Tryptophane, or indole
166	L-Tryptophane, indole, or anthranilic acid
122	Nicotinic acid

Preparation of Spores.—Potato agar medium used for the preparation of the spores of the recipient strains was prepared as follows: 200 gm. of peeled, chipped potatoes were boiled in 1 liter of tap water, filtered through cotton, and, after the addition of 5 mg. of manganese sulfate, the pH was adjusted to 6.8. The volume was then adjusted to 1 liter with tap water, 15 gm. of Difco agar was added, and the medium was autoclaved at 15 lb. pressure for 15 minutes.

Cultures (from nutrient agar slants) were heavily streaked on potato agar slants. The latter were incubated at 37° C. for 7 days and then refrigerated at 4° C. for at least 7 days. Spores were also prepared by growth on Difco Tryptose-Blood Agar Base slants for 3 days at 37° C.

Isolation and Purification of DNA.—DNA was isolated from cells of *B. subtilis* 23 grown with shaking at 37° C. in Difco Penassay broth for 18 hours. The packed cell sediment (from Sharples centrifugation) was disintegrated in a modified Hughes Press[4, 5] which had been cooled to −20° C. The crushed frozen paste was suspended in cold 0.1 *M* sodium chloride solution containing 0.05 *M* sodium citrate (approximately 10 ml. for each gram of wet cells) by homogenizing with a Ten Broeck glass homogenizer. The resulting viscous suspension was centrifuged at 30,000 × *g.* for 20 minutes at 0° C., and the supernatant was discarded. The sediment was then washed with cold 0.1 *M* sodium chloride and 0.05 *M* sodium citrate solution, and then was suspended in cold 2 *M* sodium chloride solution. After agitation with a magnetic stirrer at 4° C. for 30 minutes, the suspension was centrifuged at 20,000 × *g.* for 20 minutes. The supernatant liquid was slowly added to 2.5 volumes of cold 95 per cent ethanol, and the fibrous precipitate was rapidly removed with hooked stirring rods to avoid contamination by the subsequent fine precipitate. The fibers were dried by suction on a filter-paper pad on a Buchner funnel.

The dried fibers were suspended with the aid of a glass Ten Broeck homogenizer in cold 2 *M* sodium chloride solution. The following procedures were employed to remove the protein still associated with the DNA at this stage: After 18 hours at 4° C., sodium lauryl sulfate was added to a concentration of 0.5 per cent, and after 10 minutes at room temperature the suspension was chilled in an ice bath and

centrifuged at 4° C. at 2,500 × *g*. for 10 minutes. The supernatant fluid was then shaken with an equal volume of a chloroform-octanol (5:1) mixture for 15 minutes at room temperature and centrifuged at 2,500 × *g*. for 5 minutes. The aqueous layer was carefully aspirated from the whitish, gelatinous interface. The extractions with chloroform-octanol were repeated until no gelatinous interface could be observed, which usually required seven extractions.

The DNA was then precipitated as fibers from cold 80 per cent ethanol, dried on sterile filter paper on a Buchner funnel under suction, and restored in sterile 2 *M* sodium chloride solution. Most of the transformation studies reported in this communication were performed with the preparations purified in this manner. Sterility of the DNA preparations was essential in the transformation studies, and it was found that this was insured by the sodium lauryl sulfate and chloroform-octanol treatments.

For estimating components of the DNA preparations, the following procedures were employed. Protein determinations were made by the Folin-Phenol method of Lowry,[6, 7] using crystalline bovine albumin as standard. DNA was estimated by the diphenylamine reaction as modified by Burton,[8] using a thymus DNA preparation as standard. Ribonucleic acid was estimated by the orcinol method,[9] using *d*-ribose as standard.

Assay Procedure.—A sterile glucose minimal medium of the following composition was employed in the transformation studies:

	Per Cent
Ammonium sulfate	0.2
Dipotassium phosphate	1.4
Monopotassium phosphate	0.6
Sodium citrate ($2H_2O$)	0.1
Magnesium sulfate ($7H_2O$)	0.02
Glucose	0.5

The glucose was sterilized separately and added aseptically.

Spores of the auxotrophs from potato agar slants were suspended in glucose-minimal medium at concentrations of approximately 10^8 spores/ml, as determined by viable counting on Difco Heart Infusion Agar plates and by direct microscopic examination. The suspensions were heated at 60° C. for 5 minutes (to activate the spores for germination and to inactivate vegetative cells) and then were diluted tenfold in glucose-minimal supplemented with 0.01 per cent Difco yeast extract (2.5 ml/tube). In transformation experiments wild-type donor DNA was then added to this medium. Controls were run without DNA as well as with DNA treated with deoxyribonuclease[10] (DNAase; 2–20 μg/ml + 0.005 *M* magnesium sulfate for 30 minutes at 37° C. prior to addition to the glucose minimal). The tubes were incubated at 37° C. on a reciprocal shaker. At suitable intervals, 2–20 μg. DNAase and magnesium sulfate to give a concentration of 0.005 *M* were added and 0.1-ml. aliquots plated on glucose-minimal agar for detection of nutritionally independent transformants. In instances where additional substances were present which could support the growth of the auxotroph, the suspensions were washed with sterile 0.5 per cent sodium chloride solution before plating.

RESULTS

Demonstration of DNA Transforming Activity.—When suspensions of spores of the indole-requiring *B. subtilis* 168 were incubated with sterile preparations of

DNA isolated from *B. subtilis* 23, transformation to organisms no longer requiring indole for growth was observed. The transformation to indole independence is completely prevented by treatment of the donor DNA with 2 μg/ml or more of crystalline DNAase in the presence of Mg ions. Spontaneous reversion to non-requirement has never been observed with this strain, (i.e., in the absence of DNA from a "wild-type" donor). DNA isolated from strain 168 or from a number of *Escherichia coli* strains is inactive. As little as 2×10^{-3} μg. DNA per ml. (isolated from strain 23) produces transformation, and the number of transformants is a function of the quantity of DNA employed.

The DNA isolated from transformed cells derived from strain 168 was found to be more effective in transforming strain 168 to indole independence than the DNA derived from strain 23. Thus in the former as little as 2×10^{-4} μg. DNA per ml. had transforming activity. This represents an increase in specific activity of at least tenfold over that with DNA derived from strain 23.

Table 1 presents data from a typical experiment in which a suspension of 5×10^7

TABLE 1*

TRANSFORMATION OF *B. subtilis* 168 (INDOLE-) BY DNA FROM WILD TYPE

DNA (μg/ml)	Transformants (= Colonies on Minimal Agar per ml.) ($\times 10^3$)
20	7,000
2	4,700
0.2	2,800
0.02	480
0.002	26
0.001	12
0.0005	5.9
0.00025	2.7
0.00012	1.0
0	0
20 (+ DNAase 2 μg. + 0.005 *M* Mg)	0

* *Experimental Conditions.*—*B. subtilis* 168, 3-day growth on Tryptose–Blood Agar base suspended with shaking 4 hours at 37° C. in glucose-minimal medium containing 0.1 per cent Bacto yeast extract. Then centrifuged and resuspended in glucose-minimal medium containing 0.01 per cent yeast extract and DNA (DNA isolated from wild-type *B. subtilis* strain, produced by transformation of strain 168 with DNA from strain 23, deproteinized by chloroform-octanol extraction; RNA was not removed from this preparation) at various levels. The latter incubation was at 37° C. for 1 hour with shaking. 20 μg. of DNAase was added, and the Mg^{++} concentration was brought to 0.005 *M*. Aliquots were plated on the minimal-glucose agar medium. Colonies were counted after 24 hours. Total number of cells as determined by plating on complete medium = 5×10^7/ml.

cells per ml. of strain 168 were incubated with graded doses of DNA isolated from a wild-type strain.

Similar results have been obtained with recipient germinating spores of strain 166, which requires anthranilic acid, indole, or tryptophane for growth, and with those of strain 122 which requires nicotinic acid.

Requirements for Transformation.—Although transformation of strain 168 could be obtained in a completely defined medium (the glucose-minimal medium), the activity of the DNA was greatly enhanced by the addition of 0.01 per cent Bacto yeast extract (Difco) (Table 2). The active component(s) in yeast extract has not been identified as yet. The yeast extract supplement could not be replaced by any of the known vitamins or ribonucleotides found in yeast.

Bovine serum albumin, known to be required in *Pneumococcus* transformations,[11] is inhibitory, even in the presence of yeast extract.

TABLE 2

STIMULATING EFFECT OF YEAST EXTRACT ON TRANSFORMATION

Addition to Glucose Minimal Medium	Minimal Conc. of *B. subtilis* 23 DNA Required for Transformation of Strain 168 (18 hr., 37° C.)
None	2 μg/ml
+ 0.01 per cent yeast extract (Difco)	0.002 μg/ml
+ 0.01 per cent yeast extract + 0.04 per cent bovine serum albumin	0.2 μg/ml
+ 0.5 per cent casein hydrolyzate	2 μg/ml

Purification of DNA from B. Subtilis 23.—Analysis of the isolated fractions for DNA, protein, and RNA revealed that a combination of sodium lauryl sulfate precipitation and chloroform-octanol extractions was required for removal of protein to approximately 1 per cent or less of the DNA (Table 3).

TABLE 3

FRACTIONATION OF *B. subtilis* 23 DNA

	Fraction	DNA (μg/ml)	Protein (μg/ml)	RNA (μg/ml)
I.	First alcohol precipitate restored in 2 *M* NaCl	700	1,500	. .
II.	Supernatant fluid from sodium lauryl sulfate treatment	600	400	300
III.	Second alcohol precipitate restored in 2 *M* NaCl (after 7 chloroform-octanol extractions)	600	<10	320
IV.	Fraction III treated with RNAase (50 μg/ml), alcohol precipitated, and restored in 2 *M* NaCl solution	600	<10	<10

It can be seen from Table 4 that protein removal increased the activity 100–1,000 fold. This is somewhat surprising, since protein removal would presumably make the DNA more susceptible to cellular DNAase. On the other hand, protein removal may produce a smaller molecular species more readily able to penetrate the recipient cells.

Removal of RNA could be achieved by ribonuclease treatment (50 μg/ml of crystalline Worthington ribonuclease, 30 minutes, 37° C.) followed by alcohol

TABLE 4

TRANSFORMING ACTIVITIES OF *B. subtilis* 23 DNA FRACTIONS

	Fraction	Minimal Conc. of DNA Required for Transformation of Strain 168 (μg/ml) (18 hr., 37° C.)
I.	First alcohol precipitate restored in 2 *M* NaCl	0.2–2.0
III.	Second alcohol precipitate restored in 2 *M* NaCl (after 7 chloroform-octanol extractions)	0.002
IV.	Fraction III treated with RNAase, precipitated with alcohol and restored in 2 *M* NaCl	0.02
IV.	+ RNA fraction*	0.004

* The RNA was obtained as follows: Fraction III (5 ml. containing 600 μg/ml DNA) was treated with 50 μg/ml of crystalline DNAase and 0.005 *M* $MgSO_4$ for 30 minutes at 37° C., followed by the addition of 0.01 *M* sodium citrate. Cold ethanol was added to give a concentration of 70 per cent. A fine precipitate formed, which was collected on filter paper and was restored to the original volume with 2 *M* NaCl solution. This preparation was found to contain all the RNA originally present as determined by the orcinol reaction, but no DNA. The amount of RNA added to Fraction IV was equivalent to the original quantity present.

precipitation. Norite treatment[12] did not remove this RNA fraction, which may be closely associated with the DNA.

The presence of an RNA component appears to have potentiating activity. Its complete removal, i.e., after alcohol precipitation following ribonuclease treatment, lowers the biological activity of the DNA preparation approximately tenfold. This loss in activity can be partially restored by the addition of the RNA fraction (see Table 4).

Stability of the DNA.—The transforming activity of the purified DNA appears to be very stable in 2 *M* sodium chloride solution at 4° C. Reproducible activity has been observed over a period of months. Approximately 99 per cent of the transforming activity was destroyed by storage in dilute sodium chloride (0.1 *M*) at 4° C. for 2 days.

Transforming activity of purified DNA in 2 *M* NaCl is reduced to the extent of 99.9 per cent of the original by slow freezing at −20° C. and subsequent thawing at 37° C. On the other hand, marked stability to heat has been observed. When heated at 70° C. for 30 minutes, the minimal quantity of DNA capable of effecting transformation remained unchanged, but the number of transformants produced with any given quantity of DNA was reduced.

DISCUSSION

The present studies have demonstrated the transformation of biochemically deficient strains of *Bacillus subtilis* to nutritional independence by deoxyribonuclease-sensitive deoxyribonucleate isolated from a "wild-type" strain. Spores of the auxotrophs when allowed to germinate in a minimal medium containing glucose and 0.01 per cent of an autoclaved yeast extract could be readily transformed with as little as 2×10^{-4} μg DNA/ml. Little or no transformation occurred with the auxotrophic recipient vegetative cells actively multiplying in nutrient medium. The results obtained suggest that germinating spores may be particularly susceptible to transformation with DNA, possibly at a particular growth stage in which the cell wall is still incomplete. The DNA may be diverted in mature vegetative cells by combination with cell-wall components, such as the free amino groups of hexoseamines, diaminopimelic acid, etc. The observed presence of a DNA slime layer surrounding the cell walls of a number of bacterial species[13-14] supports this view.

The possibility that cell wall removal could allow the transfer of DNA has been explored by a number of workers. Disrupted T_2 virus which cannot infect intact cells will do so if the cell wall components are removed as in protoplasts.[15-17] Chargaff *et al.*[18] have reported the transformation of a lysine-requiring mutant of *Escherichia coli*, strain W, by the use of protoplasts of this organism and a fraction containing DNA from the wild-type strain. The small number of transformants observed may be due to poor survival as a result of the osmotic fragility of protoplasts and the complexities of cell-wall resynthesis. Lederberg and St. Clair,[19] however, have also attempted transformation of protoplasts of a number of mutants, but without success.

The activity of donor DNA appears to be increased by a ribonucleic acid (RNA) moiety closely associated with the DNA. One possible mechanism by which the RNA exerts this effect may be its activity as a specific inhibitor of deoxyribonuclease.[13a, 20, 21]

Of considerable interest is the large increase in activity of transforming DNA

achieved by protein removal. The protein remaining after detergent treatment is apparently closely associated with the DNA and may prevent easy entrance into the recipient cells by maintaining a large molecular complex.

SUMMARY

Three auxotrophic mutants of *B. subtilis* have been transformed to nutritional independence with deoxyribonucleate (DNA) isolated from a wild-type *B. subtilis*, strain 23. As little as 2×10^{-4} μg. of DNA effects transformation of heat-treated spores allowed to germinate in a glucose-minimal medium. The number of transformants produced is directly related to the quantity of DNA added and transformation is prevented by treatment with deoxyribonuclease. The specific activity of the donor DNA is increased by removal of protein, and it also appears that a ribonucleate fraction, closely associated with donor DNA, enhances transformation activity. An unidentified factor in yeast extract also appears to be required for efficient transformation. The effective DNA is relatively labile to freezing and storage in solutions of low ionic strength but is relatively stable in 2 *M* NaCl solutions.

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